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THE KINDS OF BACTERIA CONCERNED IN THE SOURING OF MILK

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE OGDEN GRADUATE SCHOOL OF SCIENCE IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

(DEPARTMENT OF BACTERIOLOGY)

BY
PAUL GUSTAV HEINEMANN

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THE SIGNIFICANCE OF STREPTOCOCCI IN MILK.*

PAUL G. HEINEMANN.

(*From the Bacteriological Laboratory, University of Chicago.*)

OF late years attention has been frequently called to the presence streptococci in milk. Von Lingelsheim[1] mentions the fact that streptococci are found in market milk, and suggests that these bacteria may be the cause of many cases of infantile intestinal diseases. Streptococci in milk have also been reported by Conn,[2] Conn and Esten,[3] Bergey,[4] Barthel,[5] Reed and Ward,[6] who also found them in the udder, and others, and special stress has been laid on the significance of leucocytes in market milk if streptococci are present. An examination by the writer of fresh milk of the highest character, in the summer of 1905 resulted in finding that streptococci are invariably present even in milk obtained with unusual precautions and from healthy cows.

This led to an investigation of the relation between the streptococci and the lactic acid bacteria ordinarily concerned in the souring of milk, it being known, among other things, that streptococci can produce and withstand a large amount of acid.

Pasteur[7] was the first to describe lactic acid bacteria. His "levure

*Received for publication, January 5, 1906.

[1] KOLLE UND WASSERMANN, *Handbuch der pathogenen Mikroorganismen*, 1903, 3, p. 337.

[2] *Centralbl. f. Bakt.*, Abth. 2, 1902, 8, p. 442.

[3] *Ann. Report Storrs Agric. Exp. Station*, 1902-3, p. 63.

[4] *Dept. of Agriculture, Commonwealth of Penna., Bull. 125*, 1904, p. —.

[5] *Milchzeitung*, Leipzig, 1903, pp. 40, 41, 42.

[6] *Centralbl. f. Bakt.*, Abth. 1, 1903, 30, p. 83.

[7] *Ann. de chim. et phys.* Série 3, 1858, 52, p. 404.

lactique" consisted of "small globules or very short particles resembling certain kinds of amorphous precipitates. They are constricted in the middle, occur isolated or in irregular clusters. They ferment lactose with acid and gas formation, and cultures are often viscid." In 1878 Lister[1] isolated an organism from sour milk by the dilution method which he called *Bacterium lactis*, a detailed description of which could not be found. In 1884 Hueppe[2] described his *B. acidi lactici*, which he found in large numbers in all samples of milk examined, and which was subsequently accepted as the lactic acid organism *par excellence*. This organism so far as can be gathered from the original description, is to be considered as a variety of *B.* [*lactis*] *aërogenes*, Escherich. Following this discovery many varieties of lactic acid bacteria have been described, some of which may be identified with the one discovered by Hueppe. Clauss,[3] Grotenfelt,[4] Schardinger,[5] Kozai(2)[6] and Utz (2),[7] have isolated lactic acid bacteria which apparently are identical with, or at most varieties of, *B. aërogenes*. The luxuriant growth on all media is characteristic. On agar, in ordinary broth, on potato, and in proteid-free media, there is abundant growth. Dextrose and lactose are fermented with acid and gas formation; milk is coagulated more or less rapidly, the coagulum contracts characteristically and whey is separated. Colonies on agar or gelatin are large, moist, and often viscid. Stab cultures produce good growth on the surface and in the puncture.

A wholly different class of lactic acid bacteria has been described by Leichmann,[8] Günther and Thierfelder,[9] Kozai (1),[10] Utz (1),[11] Esten,[12] Schierbeck,[13] and others. Günther and Thierfelder identify their organism erroneously with Hueppe's. These bacteria have been usually described as bacilli, but regarded as distinct from the *B. aërogenes* type. Leichmann was the first to call attention to this difference and unfortunately named his organism *B. lactis acidi* in distinction from *B. acidi lactici*, Hueppe. This appellation has been the cause of great confusion.

[1] *Quarterly Jour. of Microscopical Science*, 1878, 18, p. 177.
[2] *Milth. a. d. kaiserl. Gesundh. Amt.*, 1884, 2, p. 309.
[3] *Inaug. Dissert.*, Würzburg, 1889.
[4] MIGULA, *System der Bakterien*, 1900, 2, p. 544.
[5] *Monatshefte f. Chemie*, 1890, 11, p. 544.
[6] *Ztsch. f. Hyg.*, 1899, 31, p. 337.
[7] *Centralbl. f. Bakt.*, Abth. 2, 1904, 11, pp. 600 and 733.
[8] *Ibid.*, 1896, 2, p. 777.
[9] *Arch. f. Hyg.*, 1895, 15, p. 164.
[10] *Loc. cit.*
[11] *Loc cit.*
[12] *Ann. Rep. Storrs Agric. Exp. Station*, 1896
[13] *Arch. f. Hyg.*, 1901. 38, p. 294.

The experiments reported in this paper comprise first a comparative study of cultural characteristics of the following organisms:

Four lactic acid bacilli, true bacilli, one of which was in the laboratory collection labeled *B. acidi lactici;* the other three were isolated from loppered milk. These all proved to be of the *B. aërogenes* type.

Eight lactic acid organisms received from the following laboratories under the name *B. acidi lactici:* One from Professor Russell, University of Wisconsin; three strains from Professor Harding, N. Y. Agric. Exp. Sta., Geneva, N. Y.; one from Professor Prescott, Mass. Institute of Technology, Boston, Mass.; one from Professor Marshall, Michigan Agricultural College; one from Professor Bouska, Ames Agricultural College, Iowa; and one from Professor Conn, Wesleyan University, Middletown, Conn. All of these were found to belong to the group *B. lactis acidi,* Leichmann.

In addition a streptococcus from Chicago milk, isolated in this laboratory by Dr. Harris in February, 1904, was obtained for this investigation in May, 1905, and a streptococcus isolated from sewage also by Dr. Harris. Five pathogenic streptococci isolated from cases of scarlet fever, tonsilitis, and phlegmon, by Dr. Weaver, Memorial Institute of Infectious Diseases, Chicago, in February, 1904, were received for this investigation in May, 1905.* Streptococci were then isolated by the writer from fresh milk, sour milk, separator slime, market milk, cow feces, and from drippings from the udder during the process of washing previous to milking. These latter agreed well with each other and also with the so-called *B. lactis acidi.* The experiments were carried on in duplicate, one set being kept at 37° C., the other at room temperature.† Except for the fact that growth, in the majority of cases, was somewhat better at 37° C. than at room temperature, no noteworthy difference was brought out by these different temperatures.

The bacilli of the *B. aërogenes* type are all negative to Gram's stain, are generally plump, oval, and in irregular clusters. Diplobacillus forms are not infrequent. Two have capsules, one is viscid

*I wish to acknowledge my gratitude to those who have kindly supplied me with cultures for this work.

†Media were prepared according to the methods prescribed by the Bacteriological Committee of the Amer. Pub. Health Assoc. (*Jour. Infect. Dis.*, 1905, Supplm. No. 1) and 2 per cent lactose added to agar, broth, and gelatin. The formula recommended by Jordan (*Botan. Gazette*, 1899, 27, p. 1) was employed for non-proteid media. Bacteria were isolated from milk by the usual plate method with lactose-litmus agar.

in cultures, and gas is formed by all from dextrose and lactose. On agar and potato and in gelatin and non-proteid media, with and without lactose, growth is luxuriant. The colonies are well developed on the surface, are large, moist, and viscid. No difference from typical *B. aërogenes* could be noted.*

A striking similarity was found to exist between bacteria of the streptococcus and the *B. lactis acidi*, Leichmann types. The characteristics of these are briefly as follows:

Morphology.—Cocci, sometimes oval, mostly in pairs, and often with pointed ends frequently in chains of three to six members, and sometimes in chains of considerable length. Cultures in lactose broth showed chain formation to best advantage. The cocci are positive to Gram's stain.

Culture media.—Growth on slant agar and potato and in plain broth is uniformly poor, considerably better if lactose is added to media. On slant agar a delicate ribbon-like layer is formed, which often consists of single or confluent colonies, and in a few cases is hardly visible. On the surface of stab cultures there is a barely visible growth or none at all; in the puncture, however, it is quite fair. Coagulation of milk varies, from one to fourteen days being required. Litmus, if added to milk, is decolorized by *B. lactis acidi*, and all streptococci in the same typical manner. The solid coagulum turns white leaving a pink ring at the top, which gradually extends toward the bottom, and little or no whey is liberated. In non-proteid media there is no noticeable growth.

Colonies.—Small, irregular, appear mostly in the depths, but few on the surface.

The next inquiry concerned the variability of coagulative power. Experiments were conducted by passing each of the above-mentioned organisms, 33 in all, through eight tubes of litmus milk, successively. The milk employed was mixed previous to tubing and sterilizing, to insure uniform composition. Transfers were made every other day. The majority of streptococci, especially pathogenic ones, gained in coagulative power. The same was observed in streptococci from cow feces and sewage. The appearance of the coagulum of all streptococci and *B. lactis acidi*, Leichmann type was alike—showing the typical decolorization and the red ring on top.

The opinion of Houston,[1] that streptococci in water are of value in detecting sewage contamination, has received some support.[2] The

* The rules of nomenclature possibly demand that "*B. acidi lactis*, Hueppe" be adopted in place of *B. lactis aërogenes* on account of priority. As this would undoubtedly lead to confusion it seemed best for the present to retain the name "*B. aërogenes.*"

[1] *Supplm. to the 29th Annual Rep. of the Local Gov. Board*, containing the *Rep. of Med. Officer for 1899–1900*, p. 458.

[2] WINSLOW AND HUNNEWELL, *Jour. Med. Res.*, 1902, 3, p. 502; PRESCOTT AND BAKER, *Jour. Infect. Dis.*, 1904, 1, p. 193.

method employed is to inoculate dextrose or lactose broth with definite amounts of the water in question. Bacteria of the Colon-Aërogenes group multiply rapidly, and when a certain amount of acidity is reached, streptococci, being able to resist a higher degree of acidity, crowd other bacteria out. Judging from analogy it is reasonable to assume that similar conditions obtain in milk. Three lines of research were carried on to establish this view.

(1) One quart of certified milk was divided into two parts and placed in two flasks. One of these was kept at 37° C., the other at room temperature. Dilutions were prepared daily for eight consecutive days and 1 c.c. of each dilution inoculated into each of five lactose broth tubes. These were examined after three days, and streptococci appeared in all instances in high dilutions. By this method the presence of streptococci even when they occur in very small numbers can be readily demonstrated. Dilutions prepared from fresh milk showed streptococci in chains of immense length. Litmus milk inoculated from these tubes coagulated in typical fashion in 24 to 48 hours, but the long chains had disappeared, and in their stead, diplococci and short chains of three to six members appeared. As the acid fermentation progressed, the long chain formation became more and more scarce, giving way to diplococcus and short chain formation. It appears from this experiment that long chains disappear if these lactic streptococci are cultivated in milk. (See plates.)

(2) Plates in lactose litmus agar were prepared for eight successive days from two flasks of milk from the same lot, undergoing spontaneous fermentation, one at 37° C. the other at room temperature. An approximate enumeration of the colonies of *B. aërogenes* and "*B. lactis acidi*," shows that at initial stages of fermentation *B. aërogenes* multiplies more rapidly than the other type; at later stages, however, the Leichmann type gains ascendency. This phenomenon is more pronounced at room temperature than at 37° C.

(3) A third series of experiments was of the following nature: Ten liters of milk were mixed and evenly distributed in 42 flasks. Thirty-eight of these were sterilized in the Arnold for three successive days, and allowed to stand at room temperature for six days, before being used. The other four flasks were allowed to lopper, two at 37° C., the other two at room temperature. One of the sterilized flasks was

500 C.C. FLASKS OF STERILE MILK INOCULATED WITH PURE CULTURES, VARIOUS MIXTURES OF LACTIC ACID BACTERIA, SOUR MILK AND FLASKS WITH NATURALLY SOURED MILK.

Number of Flask	Inoculated with	Odor	Coagulation			Acidity per Cent in Lactic Acid		Number of Days after Which Highest Acidity Was Reached
			In Days	Homogeneous	Broken up and Whey	At Time When Coagulation Was First Observed	Highest Acidity Reached	
1....	Control	.....	....	....	....	.....	0.164	1
2....	Pathogenic streptococcus	Sour mild	5	+	—	0.507	0.726	1
3....	" "	" "	5	+	—	0.508	0.773	11
4....	Lactic acid bacterium from sweet milk	" "	3	+	—	0.664	1.024	13
5....	Lactic ac. bact. from separator slime	" "	1	+	—	0.716	1.040	5
6....	Bacillus from sweet milk	Sour and Cheesy	3	—	+	0.541	1.006	11
7....	Bacillus from sour milk	" "	3	—	+	0.619	0.816	8
8....	B. acidi lactici, University of Chicago	" "	4	—	+	0.669	1.149	10
9....	1 c.c. sour old milk for inoculation	" "	1	—	+	0.707	1.130	6
10....	Lactic acid bacter. from N. Y. Station	Sour mild	4	+	—	0.532	1.056	9
11....	Lactic acid bacterium from teat wash water	" "	1	+	—	0.557	0.969	5
12....	Streptococcus from cow feces	" "	7	+	—	0.576	0.790	13
13....	Liquefying streptococcus from milk	Indifferent	2	—	+	0.611	1.141	6
14....	Lactic acid bacterium from separator slime	Sour mild	2	+	—	0.637	0.928	6
15....	Fresh cow feces	Sour and cheesy	2	+	—	0.637	1.065	5
16....	" " "	" "	3	—	+	0.621	1.065	5
17....	1 c.c. milk after 5 days at 37°	" "	3	—	+	0.655	1.240	8
18....	1 c.c. milk after 2 days at room temp.	" "	2	+	—	1.013	1.169	8
19....	Sewage streptococcus	Sour mild	3	+	—	0.532	1.018	8
20....	Pathogenic streptococcus	" "	5	+	—	0.699	0.995	10
21....	" " "	" "	5	+	—	0.611	1.135	12
22....	B. aërogenes 1, University of Chicago	Sour mild	5	+	—	0.646	1.014	13
23....	B. coli A	Sour	5	+	—	0.607	1.310	12
24....	B. aërogenes from milk	Sour and cheesy	4	—	+	0.594	1.096	8
25....	Lactic acid bacterium (Russell)	Sour mild	6	+	—	0.628	0.778	12
26....	" " " (Prescott)	" "	7	+	—	0.546	0.546	7
27....	" " " (Marshall)	" "	3	+	—	0.568	0.820	8
28....	" " " from sour milk	" "	3	+	—	0.542	0.734	7
29....	# 24+28	Sour	2	—	+	0.546	1.057	9
30....	B. aërog. + Lact. acid bact. from milk	"	2	—	+	0.546	0.865	9
31....	B. aërog. (22)+ Lactic acid bact. from sour milk	"	2	—	+	0.620	0.987	10
32....	B. coli A + 28	Sour and cheesy	3	+	— whey	0.681	0.912	6
33....	B. aërog. + sewage streptococcus	Sour	3	—	+	0.599	0.925	11
34....	Milk at room temp.	"	2	+	— whey	0.921	1.175	5
35....	Milk at 37°	Cheesy	1	—	+	0.995	3.033	8
36....	Milk at room temp.	Sour	2	+	— whey	0.908	1.196	7
37....	Milk at 37°	Cheesy	1	—	+	0.764	2.846	8
38....	Pathogenic streptoc. at room temp.	Sour mild	2	+	—	0.570	0.691	7
39....	Lactic acid bacteria from separator slime at 37°	Sour mild	2	+	—	0.820	0.873	4
40....	Bacillus from sweet milk at 37°	Sour and cheesy	6	+	— whey	0.568	0.707	8
41....	# 21+6	Sour and cheesy	2	—	+	0.570	0.672	4
42....	# 5+6	Sour and cheesy	2	+	—	0.550	0.725	9

retained as control, the others were inoculated with various pure cultures and mixtures. Five pathogenic streptococci, a streptococcus from sewage, a streptococcus from cow feces, and eleven lactic bacteria of the Leichmann type (the latter including five varieties obtained from different laboratories), four varieties of *B. aërogenes* from milk and one from the laboratory collection, and a culture of *B. coli* recently isolated from human feces, were inoculated in pure culture in sterile flasks. Several flasks were inoculated with mixtures of pure cultures of each group, two with suspensions of cow manure, and three with sour milk.

A study of the results which appear in the accompanying table, demonstrates that the pathogenic streptococci (2, 3, 20, 21), the streptococci from sewage (19) and cow feces (12), the lactic acid bacteria from Professors Harding (10), Marshall (27), Russell (25), Prescott (26), and the lactic acid bacteria from milk (4, 28), separator slime (5 and 14), and teat wash water (11) produce fermentations in milk, which are identical. The coagulum is homogeneous, no whey appears, and the odor is agreeably, but faintly, sour. The range of time required for coagulation varies, but it has been shown that a sojourn in milk increases the power of rapidly coagulating milk.

Bacilli (6, 7, 8, 22, 23) produce a coagulum, which contracts or breaks up and liberates larger or smaller quantities of whey, accompanied by gas formation. The odor is decidedly sour and cheesy.*

Sterile milk, inoculated with sour milk, ferments like naturally soured milk, and produces the same variation of appearance. Suspensions of cow feces (15 and 16) also produce fermentations, which are indistinguishable from naturally soured milk. Occasionally liquefying streptococci are met with (13), which produce high acidity and at the same time dissolve the caseïn.

All flasks were titrated for acidity up to the maximum point, i. e., when three successive titrations gave the same result. The highest acidity was reached by naturally soured milk at 37° C. Milk soured at room temperature, and sterilized milk inoculated with mixtures and feces, fermented at approximately the same ratio. Streptococci

* Leichmann has shown that his lactic acid bacterium produces almost pure lactic acid, Hueppe's bacillus (*B. aërogenes*) produces large amounts of volatile acids, hence the difference in odor (*Centralbl. f. Bakt.*, Abth. 2, 1896, 2, p. 777).

of pathological, fecal, and milk origin form a class very similar among themselves in regard to results.

An interesting fact was brought out by comparing the results of the activity of *B. aërogenes* from milk and a mixture of the same organism with a lactic acid bacterium of the streptococcus type. The bacillus alone produced a finely granular precipitate and abundant gas. The mixture produced much less gas, the gas evolution ceasing after a short period of time. The bacillus was crowded out by the non-gas forming streptococcus and thus gas evolution stopped. Under natural conditions both types of lactic acid bacteria may be concerned in the acid fermentation of milk. Leichmann[1] has called attention to this phenomenon by stating that he isolated the Hueppe type from superficial layers where oxygen is readily obtained, the Leichmann type from deep parts of fermenting milk, the latter preferring anaërobic conditions.

That lactic fermentation varies is a matter of everyday experience. Without apparent cause the condition of the coagulum and the amount of whey separated varies in milks of excellent character. This investigation shows that typical lactic acid fermentations can be produced by inoculation of sterilized milk with lactic acid bacteria of either group, or by a mixture of both. The fermentation proceeds in a substantially similar manner as in the natural process, in spite of the chemical and physical alterations in milk which are unavoidable during sterilization. The various appearances of acid fermentations are brought about by various species of bacteria which may bear different numerical relations to each other, and by the variable degree of multiplication according to the temperature at which fermentation takes place. There is, however, a higher degree of acidity produced in naturally soured milk than in sterilized milk inoculated with these two common lactic acid bacteria either in pure culture or in mixtures. This shows clearly that other influences are at work, and in this connection Marshall[2] has pointed out that other bacteria, especially peptonizing bacteria, exert a favorable influence on the development of lactic acid bacteria.

The lactic acid bacteria of Leichmann, Günther and Thierfelder, Kozai (1), Utz (1), Esten, Schierbeck, and others are described by these writers as follows:

[1] *Loc. cit.*

[2] *Centralbl. f. Bakt.*, Abth. 2, 1902, 9, p. 313.

They are short rods (?) somewhat more than half as broad as long, are oval or frequently with pointed ends, occur in pairs and chains, and are positive to Gram's stain. They are non-motile, do not liquefy gelatin, have neither spores nor capsules. They ferment dextrose and lactose with formation of acid but not of gas. They grow sparingly in ordinary media, much better if dextrose or lactose is added. In stab cultures there is little or no growth on the surface. They grow well in absence of oxygen, some preferring anaërobic conditions. They acidify milk rapidly, form a homogeneous coagulum and decolorize litmus. In non-proteid media no growth is observed. Colonies are small, shapeless and appear preferably in the depth of the jelly.

The characteristics are in perfect accord with those resulting from the studies described. When lactic acid bacteria of the Leichmann type are compared with pathogenic, fecal, or sewage streptococci, it is impossible to detect a difference as far as cultural characteristics have been observed. A careful perusal of v. Lingelsheim's summary of characters of streptococci in Kolle and Wassermann's *Handbuch* demonstrates conclusively that there is no salient difference between recognized streptococci and lactic acid bacteria of the Leichmann type. It may, then, be confidently asserted that there is no organism known which deserves the names *B. acidi lactici* or *B. lactis acidi*. We can divide the common lactic acid bacteria into two groups. The first group are true bacilli, those described by Pasteur, Hueppe, Clauss, Grotenfelt, Schardinger, Kozai (2), and Utz (2). They are varieties of *B. aërogenes* and may be termed *B. aërogenes var. lacticus*. The second group includes the lactic acid bacteria described by Leichmann, Günther and Thierfelder, Kozai (1), Utz (1), Esten, Schierbeck, and others—all these are called bacilli by the authors—and Grotenfelt's, Weigmann's and Freudenreich's streptococci.[1] Kruse[2] has suggested the relation of the latter group to streptococci although no experimental evidence is reported, and proposed the appellation *Streptococcus lacticus*.

A general summary of the results leads to the following conclusions:

1. *Bacillus acidi lactici* is a myth. The ordinary bacteria producing lactic acid fermentation in milk are *B. aërogenes var. lacticus*, and *Streptococcus lacticus*. The possibility of *B. coli* and other forms participating in milk fermentation is not excluded.

2. *Streptococcus lacticus* agrees in morphological, cultural, and coagulative properties with pathogenic, fecal, and sewage streptococci.

[1] See MIGULA, *System der Bakterien*, 1900, 2, pp. 18, 39, 42.
[2] *Centralbl. f. Bakt.*, Abth. 1, 1903, 34, p. 737.

3. *Streptococcus lacticus* can be detected in cow feces, on the external surfaces of cows, and in milk at all stages of subsequent handling.

4. Souring of milk is caused by co-operation of both groups of lactic acid bacteria and may be aided by some peptonizing bacteria always present in market milk.

5. Gas is produced by *B. aërogenes var. lacticus*, but as a rule this organism is held in check and ultimately stopped by the presence and final ascendency of *Streptococcus lacticus*.

6. Acid is produced during lactic acid fermentation of milk by both classes of organisms to a marked degree. *B. aërogenes var. lacticus* is more sensitive to the presence of acid than *Streptococcus lacticus*. This results in the presence of *B. aërogenes* in large numbers in initial stages of fermentation, *Streptococcus lacticus* becoming master of the field in terminal stages.

7. Artificial lactic acid fermentation in sterilized milk can be produced by inoculation of pure cultures of bacteria of either group, or better by the two groups combined.

8. Since *Streptococcus lacticus* is invariably present in market milk and in fresh milk collected with good precautions, the sanitary significance of streptococci in market milk will need further investigation.

In conclusion I wish to thank Professor Edwin O. Jordan, under whose direction this work was carried on, and Dr. Norman Mac L. Harris for valuable advice and encouragement during the progress of the work.

DESCRIPTION OF PLATES.

PLATE 3.

Streptococcus pyogenes from tonsil in scarlet fever.

FIG. 1.—From serum broth.
FIG. 2.—From lactose broth.
FIG. 3.—From litmus milk.

PLATE 4.

Streptococcus lacticus from the Massachusetts Institute of Technology.

FIG. 1.—From serum broth.
FIG. 2.—From lactose broth.
FIG. 3.—From litmus milk.

PLATE 5.

Streptococcus lacticus from Chicago milk.

FIG. 1.—From serum broth.
FIG. 2.—From lactose broth.
FIG. 3.—From litmus milk.

PLATE 3.

FIG. 1.

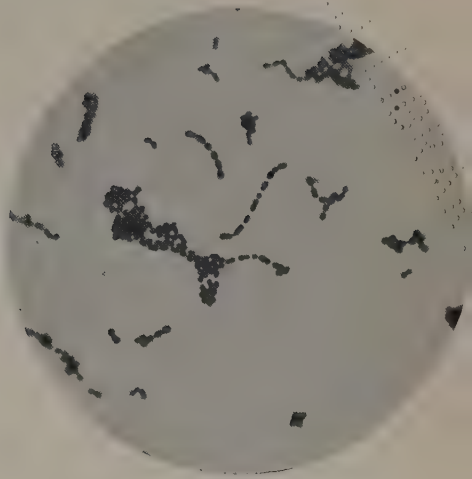

FIG. 2.

FIG. 3.

PLATE 4.

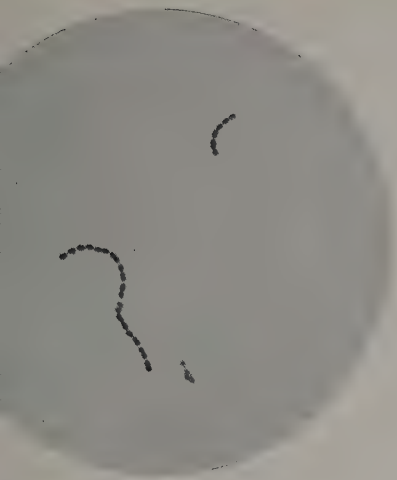

FIG. 1.

FIG. 2.

FIG. 3.

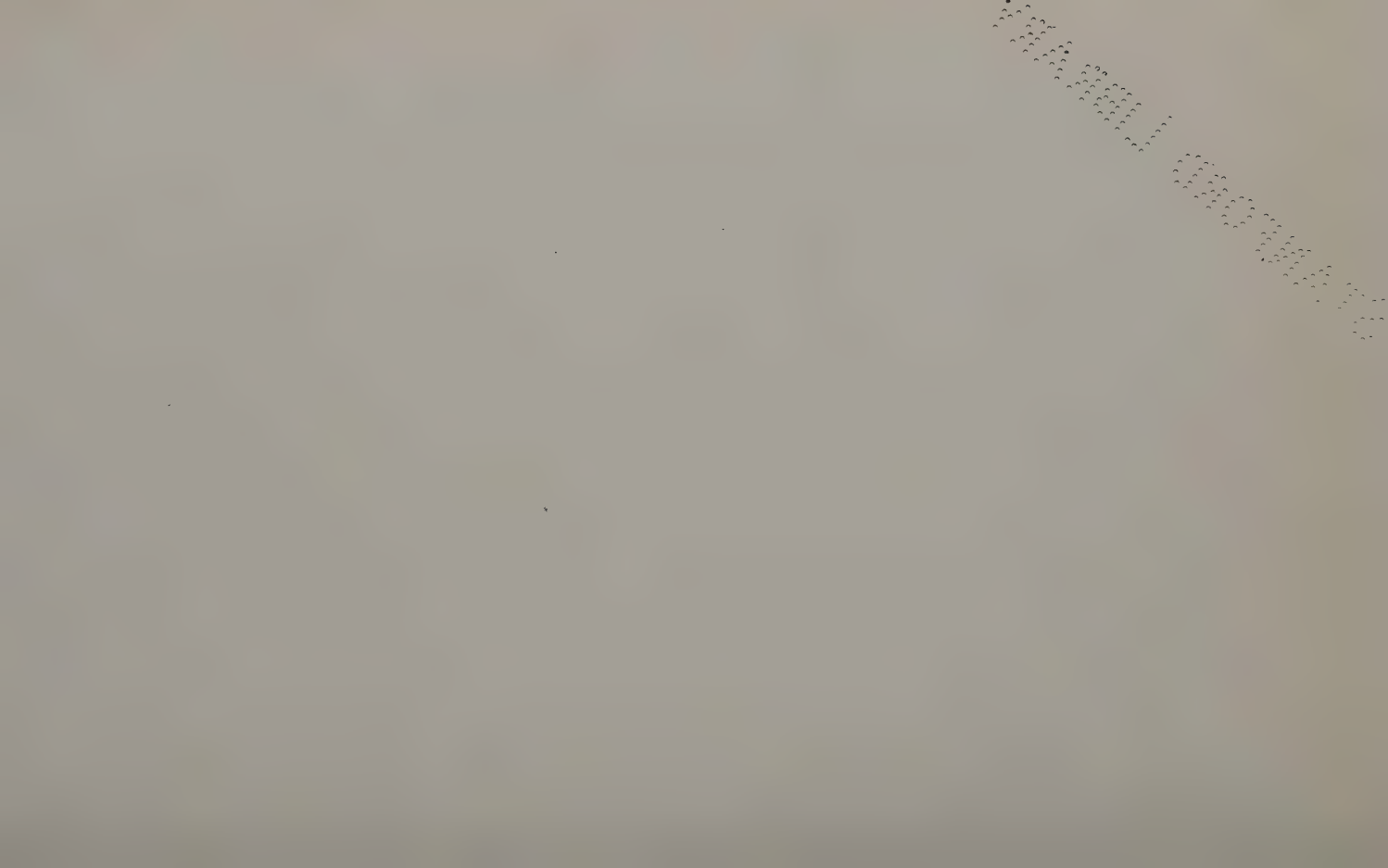

PLATE 5.

FIG. 1.

FIG. 2.

FIG. 3.

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THE PATHOGENICITY OF *STREPTOCOCCUS LACTICUS*.*

P. G. HEINEMANN.

(*From the Bacteriological Laboratory, University of Chicago.*)

IT has been shown by many observers that streptococci frequently occur in milk and Escherich[1] is of the opinion that infants fed on cow's milk have especially large numbers of streptococci in the intestines. These streptococci he found fatal to mice in some instances. Milk streptococci have been found pathogenic by some but not all observers. Beck[2] injected the centrifugal residue of milk into guinea-pigs which then died of peritonitis caused by streptococci. Pure cultures of this streptococcus in mice brought about a fatal general infection. Rubbing on the ear produced reddening of the skin in some instances. Bergey[3] injected streptococci from milk into the skin of the ears of rabbits with the result that slight local lesions developed. The amount injected is not stated. Lammeris and Harrevelt[4] recovered a streptococcus from milk which had been the cause of serious stomach troubles and diarrhea in adults, but found it non-pathogenic for rabbits and guinea-pigs. Park and Holt[5] stated that 40 per cent of all bacteria injected into guinea-pigs proved pathogenic, but whether streptococci were among these is not stated. V. Freudenreich[6] inoculated rabbits with streptococci from milk with negative results. Hölling[7] inoculated 11 mice with as many strains of *Strept. lacticus*. Three of these died. Brüning[8] found streptococci in 93 per cent of Leipzig milk which produced fatal general infections in mice but did not affect guinea-pigs. Savage[9] obtained negative results from milk streptococcus

* Received for publication October 20, 1906.

[1] *Jarhb. f. Kinderheilk.*, 1899, 49, p. 137.

[2] *Deutsche Vierteljahrsschr. f. öffentl. Gesundheitspfl.*, 1901, 32, p. 430

[3] *Am. Med.*, 1901, 1, p. 122..

[4] *Zeitschr. f. Fleisch u. Milch Hyg.*, 1901, 11, p. 114.

[5] "Upon the Results with Different Kinds of Pure and Impure Milk in Infant-feeding in Tenement Houses, etc.," *Archiv. of Pediatrics*, 1903.

[6] *Centralbl. f. Bakt.*, Abth. 2, 1904, 13, p. 281.

[7] *Inaug. Dissert.*, Bonn, 1904.

[8] *Jahrb. f. Kinderheilk.*, 1905, 12, p. 1.

[9] *Jour. Hyg.*, 1906, 6, p. 123.

injections. Müller[1] stated that the speed of coagulation of milk streptococci and pathogenic streptococci differs materially, but that there is no difference in agglutination or hemolysis. Kaiser[2] found streptococci in 76.6 per cent milk after ruling out diplococci and short chains.

It is probably true, then, that streptococci freshly isolated from milk, apart from those originating from mastitis, do not possess high virulence. I have shown[3] that *Strept. lacticus* occurs constantly in milk and since the morphological and cultural characters of this variety of streptococcus on ordinary media are identical with those of *Strept. pyogenes*, it seemed likely that the virulence might be increased by successive passages through animals.

Experiments along this line have been carried on by the writer with rabbits, the rabbit being chosen because of its susceptibility to streptococcus infection.[4] The following plan was pursued. Various cultures of *Strept. lacticus* were "rejuvenated" by passing through three tubes of serum broth, and 2 c.c. of the last culture injected, either subcutaneously or intravenously, into a rabbit. From the lesions produced the organism was recovered after two days, and 2 c.c. of a 24-hour-old broth culture then injected into another animal. From each of the animals cultures were prepared and inoculated into ordinary broth and 2 per cent lactose broth, litmus milk, and slant agar, in order to find whether any cultural difference from the original appeared. Microscopical examinations were also made in each instance. The following strains were employed:

1. *Strept. lacticus* isolated from certified milk by the writer.

2. Another strain of the same organism. These two cultures were selected at random from a large number, and were typical of *Strept. lacticus* (*B. lactis acidi*, Leichmann).

3. *Strept. lacticus* obtained from Professor Harding, N. Y. Agricultural Experiment Station, Geneva, N. Y. This organism was to all appearances identical with the two previous ones in cultural and morphological characters.

[1] *Archiv f. Hyg.*, 1906, 56, p. 90. [2] *Ibid.*, p. 51.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 173.

[4] V. Lingelsheim in Kolle and Wassermann, *Handbuch der Pathogenen Mikroorganismen*, 1903, 3, p. 350

4. *Strept. lacticus* from a commercial starter.

5. *Strept. lacticus* also from a commercial starter. These two cultures, 4 and 5, agreed with typical *Strept. lacticus* in morphological and cultural characters.*

As controls and for comparison three strains of *Strept. pyogenes* were injected into rabbits in the same manner and in the same doses. Following are the sources of these cultures.

6. *Strept. pyogenes* isolated from the tonsil in a case of tonsilitis.

7. *Strept. pyogenes* isolated from a fatal case of peritonitis.

8. *Strept. pyogenes* isolated from a case of meningitis.†

These three strains possessed the usual cultural and morphological characters, but No. 8 coagulated milk very slowly.

After passage through animals the streptococci showed no noticeable cultural divergence from the original cultures. The only morphological difference was shown by *Strept. lacticus* No. 1, the original shape of this organism changing somewhat, and the individual cells in cultures from animals appearing rounder and smaller. Microscopical preparations from the lesions showed mostly diplococci and sometimes short chains. *Strept. lacticus* and *Strept. pyogenes* appeared alike.

A detailed description of the experiments follows:

1. *Strept. lacticus* (from milk): This strain was passed through 10 rabbits successively. A uniform amount each time (2 c.c.) was injected subcutaneously with the usual antiseptic precautions. The first rabbit developed an induration at the point of inoculation. The organism was recovered from the serous exudate after two days. The second rabbit was inoculated with a 24-hour-old broth culture of the recovered streptococcus in the same manner. It developed a small abscess. The pus was of a tough viscous consistency. The organism was recovered from this pus and a third animal inoculated with a culture therefrom. These methods of inoculation and recovery of the bacteria were pursued throughout all succeeding experiments. The pus pocket increased in size and rapidity of development with each succeeding rabbit. The fifth animal died in 29 days. Numerous pus centers were found under the skin, and purulent exudates were seen in the pleural and pericardial cavities. The ninth rabbit died in 16 days from a general streptococcus infection. There were fibrino-pur-

* Seven commercial starters were obtained from the manufacturers and examined bacteriologically. Five of these consisted of practically pure cultures of *Strept. lacticus*, while two were not pure cultures but contained streptococci in large numbers After preparing pure cultures from all starters by plating, they were studied and found to possess all the characters of *Strept. lacticus*. The streptococci obtained from two of the starters were very feeble, growing but poorly and coagulating milk much more slowly than *Strept. lacticus* usually does.

† These strains were kindly given me by Dr. H. T. Ricketts and Mr. J. E. Tyree, Department of Pathology, University of Chicago. They were isolated less than two weeks before the commencement of my experiments

ulent exudates in the pleural, pericardial, and peritoneal cavities, and numerous purulent foci under the skin. Streptococci were recovered from the pleural and peritoneal cavities, heart's blood, kidney, spleen, and subcutaneous pus. The 10th rabbit died after nine days. The lesions were similar to those described in the foregoing. All these rabbits lost progressively in weight and presented an emaciated appearance. Five out of the 10 rabbits inoculated gradually recovered. Several, at the time of writing, still have a subcutaneous pus pocket and their emaciated appearance gives rise to the conjecture that they may finally die. In two instances no lesions developed at the point of inoculation. These rabbits died after a lapse of four weeks with the phenomena of general infections of the same nature as described, except that no noticeable change took place at the point of inoculation.

The same streptococcus (No.1) from the original culture was injected intravenously into a rabbit. The animal lost weight slowly and finally died after 25 days A large subcutaneous pus pocket had formed in the neighborhood of the hip on the right side, coincident with numerous pus foci of the same nature in other parts of the body. The pleural and peritoneal cavities were filled with a sero-fibrinous exudate and the pericardium showed a fibrino-purulent exudate. The organism was recovered from the pus, pleural, peritoneal, and pericardial cavities, and the heart's blood. From a broth culture prepared from the heart's blood a normal rabbit was injected intravenously and likewise a rabbit of the same weight, which had been previously inoculated subcutaneously with the same organism, but had recovered from the effects to all appearances. The former died in 17 days, the latter in six. The lesions were the same in both cases and identical with those described.

2. *Strept. lacticus* (No. 2, from milk): This strain produced no local effect. The animal after several days commenced to carry the head bent toward the left side, and when touched would walk around in a circle. When put on its back it regained its foothold only after several strenuous efforts. The animal seemed gradually to recover its normal condition, although it was considerably emaciated. Quite unexpectedly, after about six weeks, it died. Postmortem examination showed an enormously large amount of whitish semi-fluid pus in the pleural cavity. No other lesions could be detected. Streptococci were recovered from the pus and the heart's blood. Possibly the twisting of the head was due to an affection of the central nervous system by a toxin produced by the streptococcus. Similar observations have been recorded, among others by v. Lingelsheim.[1]

3. *Strept. lacticus* (from N. Y. Agricultural Experiment Station): The results of subcutaneous inoculation were quite similar to those described, excepting that the virulence increased somewhat more rapidly. The seventh rabbit died in 16 days, the eighth after 10 days.

4. *Strept. lacticus* (from a commercial starter): This organism seemed more virulent than any of the former three. The first rabbit developed a pus pocket at the point of inoculation of about an inch in diameter. The pus was of a thick, ropy consistency. An erysipelas-like reddening of the skin developed in the third rabbit at the point of inoculation. This reddening spread rapidly for four or five days, then came to a standstill, and finally disappeared. A large area of necrotic tissue formed in the fourth animal. This one also finally recovered. The seventh rabbit died on the ninth day after inoculation with the same postmortem picture as seen

[1] *Loc. cit.*

in the other animals. Here also streptococci were recovered from the pleural and pericardial cavities and the heart's blood.

5. *Strept. lacticus* (from a commercial starter): This strain was injected subcutaneously but produced no local effect. The rabbit suffered from a nervous trouble similar to that described under No. 2. The animal carried the head bent over to one side but gradually seemed to recover. It died after several weeks. No postmortem lesions whatever could be detected.

6, 7, 8. *Strept. pyogenes:* The three varieties of *Strept. pyogenes* produced the same effect on rabbits as *Strept. lacticus*, except that death occurred in a shorter time. *Strept. pyogenes* from tonsilitis (No. 6) caused the sixth rabbit to die in seven days. *Strept. pyogenes* from peritonitis caused the third rabbit to die in eight days, and *Strept. pyogenes* from meningitis produced the death of the fourth rabbit in seven days. The lesions were in every respect identical with those brought about by *Strept. lacticus*. By a fortunate coincidence the 10th rabbit inoculated with *Strept. lacticus* (No. 1) and the third rabbit inoculated with *Strept. pyogenes* from peritonitis (No. 7) died at the same time, the former after nine, the latter after eight days. Postmortem examinations were made simultaneously. The pathological conditions in the two animals were strikingly alike. Pus pockets under the skin in various places, the usual pericarditis, and the purulent exudate in the pleural cavity revealed identical conditions in both cases. Streptococci were recovered in both cases from the affected parts.

At the end of these experiments the different cultures from each of the animals were cultivated on slant lactose agar, plain and lactose broth, and litmus milk. It was noted that no change in the reactions on these media was effected by the passage through animals.

The conclusion seems justified that *Strept. lacticus*, by prolonged habitation in the body of the rabbit, is able to become virulent in large measure and approximate, if not equal, the virulence for this animal of typical *Strept. pyogenes* from human affections. It must be taken into consideration that the varieties of *Strept. pyogenes* employed in these experiments were recently isolated from pathological lesions. The streptococci from milk (Nos. 1, 2, and 3) had possibly lost in virulence by nine months' cultivation on artificial media. The organisms from commercial starters, although freshly isolated from the starters, probably had been in artificial cultivation for an indefinite length of time.

The negative results of inoculation experiments with milk streptococci by some observers may be explained in two ways. First: The lesions are often, especially in the beginning, insignificant and are easily overlooked. Second: Since lesions are insignificant with large doses it is quite possible that small doses are unable to create

any noticeable disturbances. Summarizing the results of the experiments described the following points seem established.

1. *Strept. lacticus* is closely related to *Strept. pyogenes*, not only morphologically and culturally, but also in pathogenic property.

2. By repeated passages of *Strept. lacticus* through rabbits its virulence is gradually increased. After five or more passages *Strept. lacticus* in subcutaneous injections becomes fatal in doses of 2 c.c. of 24-hour-old broth cultures. The same amount injected intravenously proved fatal to the first animal.

3. The lesions produced by *Strept. lacticus* in rabbits are of the same kind and extent as those produced by *Strept. pyogenes* from pathological conditions in human beings.

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THE KINDS OF LACTIC ACID PRODUCED BY LACTIC ACID BACTERIA.

BY P. G. HEINEMANN.

(*From the Bacteriological Laboratory, University of Chicago.*)

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The writer has called attention to the similarity of the cultural and morphological characters of B. acidi lactici (Hueppe and others) with those of B. (lactis) aërogenes (Escherich), and also to the similarity of B. lactis acidi (Leichmann and others; Streptococcus lacticus, Kruse) and Streptococcus pyogenes.[1] In a later paper a resemblance is also shown to exist between Str. lacticus and Str. pyogenes[2] in respect to their behavior when inoculated into rabbits. Additional proof of the similarity of Str. lacticus with Str. pyogenes and of B. acidi lactici with B. aërogenes may now be presented.

The results of experiments of various authors on the kind of lactic acid formed in naturally soured milk do not agree. Günther and Thierfelder[3] state that the lactic acid present in naturally soured milk is not always solely the so-called fermentation lactic acid or, as it is also termed, the optically inactive or racemic variety.[4] They found that in most cases, to be sure "inactive acid" was present, but that often there was a preponderance of dextro-rotatory acid. The organism called B. lactis acidi (Streptococcus lacticus, Kruse) in pure culture produced invariably pure d–acid. Leichmann[5] found that B. lactis acidi in pure culture produced only d–acid. He expected

[1] *Journ. of Infect. Dis.*, iii, p. 173, 1906.

[2] *Ibid.*, iv, p. 87, 1907.

[3] *Arch. f. Hyg.*, xxv, p. 164, 1895.

[4] In accord with modern chemical nomenclature the expression "racemic acid" or "r–acid" will be employed in this paper in place of "inactive" or "i–acid."

[5] *Centralbl. f. Bakt. etc.*, ii, 2, p. 777, 1896.

to find the same acid in naturally soured milk, but observed that when milk soured at higher temperatures "inactive" acid was found. According to Thiele[1] there is present mostly d–acid at room temperature during the first few days, but after seven days "inactive" acid with some d–acid is present. At 37° C. d–acid is found after 36 hours, later "inactive," and finally "inactive mixed with l–acid." At variance with these results are those of Kozai.[2] This author found in milk, naturally soured at room temperature, pure d–acid or "d–acid mixed with a small amount of inactive acid," while at 37° he found "inactive with some l–acid." He concludes that bacteria forming l–acid grow preferably at the higher temperature. The presence of "inactive" acid is attributed to the activity of two different species of bacteria. Kozai also advances the idea that possibly one of the two acids is consumed by bacteria. Nef,[3] however, has observed the auto-transformation of active lactic acid to racemic acid. Utz[4] agrees with Kozai in noting that "inactive or inactive mixed with d–acid" are produced at room temperature, but could observe no difference at higher temperatures. MacKenzie[5] has criticised fundamentally the methods used by the above mentioned observers. He argues that, since active zinc lactates are soluble in 17.5 parts of water at 15° C., while inactive zinc lactate is soluble in 53 parts, there is the possibility, that in the process of crystallizing the difficultly soluble inactive salt any active salt present may remain in solution in the mother liquor. By repeated crystallization (the zinc salt being recrystallized in order to purify it) all traces of active salt may be lost sight of. The author concludes, therefore, that the results of Günther and Thierfelder, Kozai, Utz, and Thiele are not as contradictory as might appear on the surface, since their methods probably allowed some active salts to escape observation. MacKenzie also advances the view that racemic acid is never produced by the one species of micro-organism, but that separate species produce either one or the other of the two active acids.

[1] *Zeitschr. f. Hyg.*, xlvi, p. 394, 1904.
[2] *Ibid.*, xxxi, p. 337, 1899.
[3] *Ann. d. Chem.*, cccxxxv, pp. 243 and 290, 1904.
[4] *Centralbl. f. Bakt. etc.*, xi, 2, p. 600, 1903–04.
[5] *Journ. of the Chem. Soc.*, lxxxvii and lxxxviii, p. 373, 1905.

This opinion has been confirmed by carefully executed experiments with pure cultures of various moulds by MacKenzie and Harden.[1]

My attention was called to MacKenzie's paper after the greater part of the results presented in the accompanying tables had been gathered. Towards the end of my work a few tests of the mother liquor were made and the results fully bear out MacKenzie's criticism. In several instances inactive zinc salt crystallized leaving appreciable amounts of active salt in solution.

I have carried out two series of experiments. The object of the first series was to determine what kind of acid was produced by lactic acid bacteria in pure culture and in mixtures. The list included various strains of Str. lacticus, Str. pyogenes, Micr. lanceolatus, B. acidi lactici (B. aërogenes var. lacticus), and one strain of B. aërogenes and B. coli. Cultures of these organisms were added to "certified" milk, and control plates prepared to test the purity of the cultures. After the bulk of the cream had been removed.[2] the milk was sterilized for three days in the steam sterilizer and then preserved at room temperature for one week to test the sterility. The fermentation of the milk containing pure cultures was interrupted after three days; in the flasks with mixtures the fermentation was interrupted in three days in some instances, in others after five days. The results appear in Table I. All the streptococci produced d–acid. All the bacilli produced l–acid. In mixtures kept at 37° C. those which stood for three days contained d–acid, those which stood for five days r–acid. At room temperature pure d–acid is formed.

The second series of experiments was carried out in the following manner: Samples of milk from four different sources were examined. Each of the four kinds of milk was treated as follows. After removing the bulk of the cream of 21 one quart bottles the contents were emptied into a large container and thoroughly mixed, thus securing perfect uniformity of the milk.

[1] *Proc. of the Chem. Soc.*, xix, p. 48, 1903.

[2] By removing the cream but little fat remained to dissolve in the ether and the isolation of the lactic acid was facilitated. This was of particular importance, when the coagulum was granular or incomplete.

Plates in lactose litmus agar were then prepared from this fresh milk. The counts after three days were the smallest for "certified" milk, marked "C" in the table. The next higher count was found in milk "B," then follows "A," and "M" finally contained the largest number of bacteria.

TABLE I.

SPECIFIC ROTATORY POWER OF ZINC LACTATES PREPARED FROM LACTIC ACID, PRODUCED BY PURE CULTURES AND MIXTURES OF PURE CULTURES IN STERILIZED MILK.

Organism.	At 37° C.	Room Temperature.
Pneumococcus A.	−7.33	Not sufficient growth.
" B.	−7.33	"
Str. Pyogenes. (Mening.)	−7.14	"
" " (Tonsil.)	−7.81	"
" " (Scar. f.)	−7.37	"
" " "	−7.44	"
" lactic (Chic. milk)	−7.60	−7.49
" " " "	−7.29	−7.40
" " " "	−7.31	−8.00
" " " "	−7.20	−7.58
" " " "	−7.47	−7.38
" " " "	−7.24	−7.30
" " " "	−7.31	−7.49
" " " "	−7.14	−7.56
" " (Starter)	−7.20	−7.66
" " (Russell)	−7.21	−7.55
" " (N. Y. Sta.)	−7.16	−7.08
B. ac. lact. (Hueppe)	+7.30	+7.55
" " (Chic. milk)	+7.75	+7.74
" " (Lab. U. of C.)	+7.17	+7.30
B. aërog. " " " "	+7.11	+7.20
B. coli " " " "	+7.24	+7.61
B. ac. lact. + Str. lact.	−7.47	−7.31
" " " " "	−6.62	−7.61
" " " " "	inact.	−7.05
" " " " "	"	−7.06
" " " " pyog.	−5.32	+6.05
B. coli + Str. lact.	inact.	−7.06
B. aërog. + Str. lact.	−7.23	−7.33

Each one of these four lots of mixed milk was then transferred to 18 one-liter flasks, which had been sterilized. Nine flasks of each lot were kept at room temperature, the other nine at 37° C. One flask of each lot was removed every succeeding day and the zinc lactate prepared in the usual manner. The four kinds of milk were treated in the same fashion.

TABLE II.

SPECIFIC ROTATORY POWER OF ZINC LACTATES, PREPARED FROM LACTIC ACID, PRODUCED IN NATURALLY SOURED MILK.

Kind of Milk.	Days.	At 37° C.	At Room Temperature.
"C"	1	−7.59	−7.09
	2	−7.45	−7.19
	3	−7.34	−7.23
	4	−7.43	−7.43
	5	−7.31	−7.23
	6	−1.51	−7.53
	7	inact.	−7.38
	8	"	−7.79
	9	"	−7.30
	21	no test	−5.71
"A"	1	−7.34	−7.26
	2	−6.27	−7.76
	3	−4.86	−7.09
	4	−1.38	−7.11
	5	−0.90	−7.47
	6	inact.	−1.92
	7	"	inact.
	8	"	"
	9	+1.02	"
"B"	1	−7.14	−7.32
	2	−6.18	−7.18
	3	−4.07	−7.53
	4	−2.84	−5.70
	5	−1.11	−3.15
	6	inact.	−1.02
	7	"	inact.
	8	"	"
	9	+1.98	"
"M"	1	−7.34	−6.27
	2	−4.40	−5.31
	3	inact.	−4.80
	4	+0.76	−3.49
	5	+1.17	−2.03
	6	+1.43	+2.10
	7	+1.67	inact.
	8	+2.36	"
	9	+3.46	"

Examination of the figures in Table II shows that the first lot of milk, marked "C" and which was "certified" milk of the highest character, contained pure d–acid throughout five days at 37° C., on the sixth day a mixture of d–acid and l–acid, the former somewhat in excess. Finally, on the seventh, eighth.

and ninth days, r–acid only was present. At room temperature pure d–acid was present for nine days. It happened that one flask in excess of the regular number stood for 21 days, after which time a small amount of l–acid was found to be present.

The other three kinds of milk were market milk of good average quality. Milks "A" and "B": Both kinds show similar results. At 37° C., commencing on the first day both active acids were present in equal amounts so as to form r–acid. On the ninth day l–acid was slightly in excess The mother liquor, however, retained increasing amounts of l–acid from the seventh day in "A," and l–acid was found in excess on the ninth day in "B." At room temperature d–acid was present for five and three days respectively, and r–acid on the seventh to ninth day. The mother liquor retained some d–acid in excess of r-acid for two days after the inactive zinc salt had been crystallized.

Milk "M": At 37° C. d–acid was formed on the first day, on the second there was d–acid with some l–acid, on the third r-acid, and after that the excess of l-acid increased steadily. At room temperature the increase of l–acid was much slower than at 37°C. and equalled the amount of d–acid on the seventh day without being in excess up to the ninth.

These experiments show that in milk from different sources different kinds of lactic acid may develop and that in "certified" milk the period during which d–acid predominates is longer than in ordinary milk. Preponderance of d-acid is also favored by preserving milk at low temperatures.. Lactic acid in sour milk is produced chiefly by the activity of Streptococcus lacticus and Bacillus aërogenes. Since "certified" milk contains d–acid in excess of l–acid for a longer period of time both at 37° C. and at room temperature than the other kinds of milk, it seems safe to conclude that, from a bacteriological point of view, Str. lacticus predominates in approximate proportion to the purity of the milk.

Before the whey was separated from the coagulum and filtered for the purpose of isolating the lactic acid, the flask was thoroughly shaken and one cubic centimeter diluted 1:10,000 with sterile water and one cubic centimeter of the dilution plated in lactose litmus agar. After three days of incubation the colonies were counted. The results confirmed the conclusion

arrived at in a previous paper,[1] that the number of Str. lacticus increases with the progress of the acid fermentation, the number of B. aërogenes, however, decreases. This phenomenon was also noticed at 37° C., but was not so pronounced as at room temperature. Conn[2] has found 95 to 100 per cent. of all organisms in loppered milk to be of the B. lactis acidi type (Str. lacticus). It is to be observed, however, that this increase of Str. lacticus, which produces pure d–acid, is not accompanied by a relative increase of d-acid, on the contrary, l–acid increases in proportion to d–acid together with a decrease in numbers of B. aërogenes, although this latter organism produces pure l-acid. This apparent discrepancy is difficult to explain. We find frequent mention in the literature of the fact that micro-organisms prefer assimilating one of the two optical antipodes to the other, if the racemic variety is in the medium. The greater number of these observations show that d–acid is consumed and that l–acid is left intact or attacked but little. If this be true, we must assume according to Nef's theory of dissociation[3] that d-acid is dissociated to a higher degree than l–acid and is, therefore, consumed by some of the bacteria present as Kozai has supposed or, as Nef has suggested, that by auto-transformation depending on dissociation, d–acid is converted into l–acid respectively r–acid. This view receives support from the fact that at 37° C. the relative amount of l–acid increases more rapidly than at room temperature.

Another explanation is suggested on the basis of Buchner and Meisenheimer's[4] and Herzog's[5] discovery that lactose is converted into lactic acid by the activity of an endoenzyme produced by lactic acid bacteria. This enzyme may be liberated and diffused through the medium after the death of B. aërogenes and continue forming lactic acid from the lactose present. A minute amount of enzyme is all that is necessary to produce this result. Buchner and Meisenheimer did not test the rotatory power of the lactic acid produced by their enzyme, but state that it appeared

[1] *Loc. cit.*
[2] *Fifteenth Ann. Rep. Storrs Agric. Ex. Sta.*, 1903, p. 92.
[3] *Loc. cit.*
[4] *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 634, 1903.
[5] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 381, 1903.

to be optically active. The greater increase of l–acid at 37° C. favors this view, as it is known that enzymes generally act more rapidly at temperatures higher than room temperature.

The opposing views of Leichmann, Günther, and Thierfelder on one side and of Kozai and others on the other can be harmonized by consideration of the following points:

1. It has been shown that in milk, obtained and preserved with aseptic precautions, d–acid is the chief product, both at room temperature and at 37° C. In milk of poorer quality the d-acid is rapidly neutralized by l–acid so that r-acid results and at incubator temperature the excess of d–acid is of such short duration that l–acid appears in equal amounts after forty-eight hours. Milk "M" in the table is of a quality above that of average market milk sold in bulk and it is quite in accord with the foregoing experiments to assume that milk with high bacterial content may contain r–acid in 24 hours at 37° C. In a later paper Günther and Thierfelder[1] have described experiments in which d-acid is formed chiefly at room temperature, but they found no constant variation at 37° C. The time of fermentation is not stated, but it is stated that the eight samples of milk examined were of unusually good quality. Nothing but d–acid could be expected under such conditions. The authors conclude that the kind of acid produced varies with different conditions and that they can find no adequate explanation of this phenomenon. The experiments discussed in this paper indicate that the quality of the milk examined influences the results, and the possibility of Kozai having worked with milk of a higher standard affords a reasonable explanation of the differences between his results and those of Günther and Thierfelder in their earlier work.

2. The time elapsing from the commencement of acid fermentation to the interruption has been shown to influence the results. Milk examined at an early period may contain d–acid, at a later period r–acid or even l–acid in excess. To obtain comparable results it is necessary to state the initial bacterial content, the length of time of fermentation, and the temperature at which the milk has been kept. Kozai and Thiele have examined milk at various stages of fermentation and have arrived at similar

[1] *Hyg. Rundschau*, x, p. 769, 1900.

results, viz., l–acid increases in relative amount to d–acid in approximate proportion to the period of fermentation.

It is also to be borne in mind that there are several factors which vitiate the accuracy of the results obtained by the usual method of preparing zinc lactates from sour milk. Evaporation of a solution of lactic acid or of lactates to concentration causes the formation of lactic anhydride which is lævo-rotatory and may change the specific rotation from right to left. The salts of this anhydride are highly rotatory but in the opposite direction.[1] Another source of error is the point mentioned above to which MacKenzie has called attention. If any active salt is present in a solution of inactive, the former will remain in the mother liquor when the latter crystallizes. By repeated crystallization the last traces of active salt will then be washed away and lost sight of. To what extent these sources of error may have influenced the results of the authors mentioned it is difficult to estimate. The comparability of results is probably not affected to a high degree, but it is more than likely that active salts have been frequently overlooked.

Summarizing the results of the experiments described we find that:

1. Milk naturally soured at room temperature contains chiefly d–acid. Milk soured at 37° C. contains chiefly r–acid with l–acid in excess if allowed to stand several days.

2. Streptococcus lacticus and Str. pyogenes produce the same kind of lactic acid, *i. e.* d–acid. B. aërogenes from milk (B. acidi lactici) and the ordinary laboratory strain of B. (lactis) aërogenes (Escherich) produce the same kind of lactic acid, *i. e.* l–acid.

3. The lactic acid produced in naturally soured milk varies:

(a.) According to the relative numbers of Str. lacticus and B. aërogenes present. The higher the number of B. aërogenes the more l–acid is produced.

(b.) According to the temperature at which the fermentation takes place, other conditions being equal. At 37° C. relatively more l–acid is formed than at room temperature.

[1] Jungfleisch and Godchot, *Compt. rend. de l'Acad. des sci.*, cxlii, p. 515, 1906.

(c.) According to the length of time the fermentation has lasted. The longer the time the more l–acid is formed.

4. In "certified" milk, d–acid only was present at room temperature for nine days, while both d–acid and l–acid were present in milk of poorer quality after one to four days. At 37° l–acid was apparent after six days in "certified" milk and on the second day in other milk. It seems as if the purer the milk the longer the excess of d–acid persists.

5. Racemic lactic acid is the result of the formation of pure d–acid and pure l–acid by at least two different species of micro-organisms. Racemic lactic acid is not known to be the product of one species only.

6. Since it is known that B. aërogenes forms other acids besides lactic acid, often in appreciable amounts, while Str. lacticus produces almost pure d–acid, the presence of d–acid may be taken as indicating desirable conditions for dairy work, because this shows the absence of the fermentation products of B. aërogenes, *i. e.* volatile acids, gas, and ethyl alcohol.